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Research Note

Expression of Pectate Lyase from *Colletotrichum gloeosporioides* in *C. magna* Promotes Pathogenicity

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To test the contribution of pectate lyase (PL) to promoting fungal pathogenicity, a pectate lyase gene (*pel*) from the avocado pathogen *Colletotrichum gloeosporioides,* isolate Cg-14, was expressed in *C. magna* isolate L-2.5, a pathogen of cucurbits that causes minor symptoms in watermelon seedlings and avocado fruits. Isolate L-2.5 was transformed with pPCPH-1 containing *hph-B* as a selectable marker and the 4.1-kb genomic *pel* clone. Southern hybridization, with the 4.1-kb genomic *pel* clone or 2.13-kb *hph-B* cassette as probes, detected integration of *pel* in transformed *C. magna* isolates Cm-PL-3 and Cm-PL-10. Western blot (immunoblot) analysis with antibodies against Cg-14 PL detected a single PL secreted by L-2.5 at a molecular mass of 41.5 kDa, whereas the PL of *C. gloeosporioides* had a molecular mass of 39 kDa. When PL activity was measured 4 days after inoculation in pectolytic enzyme-inducing media (PEIM), transformed isolates Cm-PL-3 and Cm-PL-10 showed additive PL activity relative to both Cg-14 and L-2.5. Transformed isolates also showed additive maceration capabilities on avocado pericarp relative to the wild-type *C. magna* alone, but did not reach the maceration ability of *C. gloeosporioides*. However, more severe maceration and damping off developed in watermelon seedlings inoculated with the transformed isolates compared with the two wild-type isolates, which showed no symptom development on these seedlings during the same period. Results clearly show the contribution of a single *pel* to the pathogenic abilities of *C. magna* and suggest that PL is a pathogenicity factor required for the penetration and colonization of *Colletotrichum* species.

Additional keywords: pectolytic enzymes.

Colletotrichum gloeosporioides, a pathogen of avocado fruits, has been found to produce endopolygalacturonase (endo-PG) (Prusky et al. 1989), pectin lyase (PNLA) (Bowen et al. 1995), pectin methyl esterase (PME) (Ortega 1996), and pectate lyase (PL) (Wattad et al. 1997) during the colonization of infected tissue. Evidence of the importance of PL secretion during *Colletotrichum* colonization on avocado fruits has been found in a number of studies: (i) reduced symptom development of a *C. magna* mutant (Path-1) with limited secretion of PL (Wattad et al. 1995); (ii) inhibition of decay following coinoculation of *C. gloeosporioides* spores with PL antibodies (Wattad et al. 1997); (iii) the activity inhibition of pectolytic enzymes by the host flavonoid epicatechin, which correlated with the inhibition of symptom development (Wattad et al. 1994); and (iv) a lack of decay development under conditions that are not permissive to PL secretion (Yakoby et al. 2000). These results suggest that PL is a limiting factor during the early stages of pathogenesis (Wattad et al. 1997; Yakoby et al. 2000).

Cell-wall-degrading enzymes (CWDEs) are considered to play a role in the pathogenesis of bacteria and fungi on their hosts (Collmer and Keen 1986; Ried and Collmer 1988; Annis and Goodwin 1997). A significant amount of work has been published on the role of pectolytic enzymes, but only a few reports have shown their clear involvement in fungal pathogenicity (Shieh et al. 1997; ten Have et al. 1998). Of the approaches that have been taken to demonstrate this involvement—(i) gene disruption; (ii) gene complementation of a deficient mutant; (iii) heterologous gene expression; and (iv) antisense gene expression—the first has been considered the most direct tool (Bowyer et al. 1995). However, when disrupted mutants are targeted in complex systems such as that of CWDEs, where several enzymes are secreted during fungal attack, the disruption of a single enzyme may be complemented by the activity of other secreted CWDEs (Hamer and Holden 1997). Indeed, most publications on disrupted CWDEs show no reduction in pathogenicity (i.e., Bowen et al. 1995; Gou et al. 1995; Sheng-Cheng et al. 1997). These reports suggest that the secretion of other CWDEs that share similar or overlapping enzymatic activities can easily overcome the loss of one enzyme. On the other hand, disruption of *pecA*, encoding the endo-PG P2c in the saprophytic fungus *Aspergillus flavus*, resulted in decreased cotton ball invasion (Shieh et al. 1997). Furthermore, the disruption of *Bcpg*1, one

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of the genes encoding PG of *Botrytis cinerea*, reduced its colonization by 22 to 50% (ten Have et al. 1998).

Heterologous expression was used to test the role of PG (*pg*1) from *Fusarium oxysporum* f. sp. *lycopersici* in a *pg*1-deficient isolate of *F. oxysporum* f. sp. *melonis*, where the PG-expressing isolate showed no change in virulence (Di Pietro and Roncero 1998). On the other hand, Dickman et al. (1989) showed that the insertion of a cutinase gene from *F. oxysporum* f. sp. *pisi* into species of *Mycospherella*, a pathogen that usually penetrates through wounds, enabled the latter's direct penetration through the cuticle. Heterologous expression was also used for complementation of the melanin biosynthetic pathway from *Alternaria alternata* in a deficient *Magnaporthe grisea* mutant, restoring the latter's pathogenicity (Kawamura et al. 1997).

To evaluate the contribution of a single enzyme to the pathogenic abilities of a fungus, we expressed a 4.1-kb clone encoding PL from *C. gloeosporioides* in *C. magna*, a weak pathogen of avocado and watermelon that colonizes both hosts (Prusky et al. 1994). Comparison of the genomic clone *pel* (partial sequence [1.52 kb] of the clone is published [accession number AF052632, GenBank]) to the *pel* cDNA clone (accession number U329242, GenBank) showed no introns in the latter. The sequence surrounding the ATG start codon, 5′ CAAG*AT*GAA 3′, closely resembles the Kozak sequence, 5′ CAMM*AT*GNC 3′, identified in the genes of filamentous fungi and higher eukaryotic genes (Ballance 1986; Kozak 1986).

A transformation vector was constructed by subcloning the 2.13-kb *hph-B* cassette (hygromycin phosphotransferase) from pHA-1.3 (Redman and Rodriguez 1994) into pGEM-7Z at sites *Xba*I and *Hin*dIII, generating pGH-1. The 4.1-kb *pel* clone, restricted with *Eco*RI, was blunt ended and subcloned into the plasmid pGH-1 at the blunt-ended *Xba*I site, generating the 9.23-kb pPCPH-1. Protoplasts of the wild-type *C. magna* isolate L-2.5 were transformed with 1 µg of pPCPH-1 and selected as previously described (Redman and Rodriguez 1994). Two transformed isolates, Cm-PL-3 and Cm-PL-10, from 20 colonies, were randomly selected for further analysis. One isolate, GH-2, transformed with pGH-1, was selected and further analyzed for the effect of hygromycin on virulence. The isolate was found to be hygromycin resistant, contained the *hph-B* cassette, and possessed the same virulence abilities as wild-type *C. magna* (data not shown).

Southern blot analysis of the transformed *C. magna* and two wild-type isolates, Cg-14 and L-2.5, showed integration of the plasmid at different sites in the genome, when probed with the 4.1-kb *pel* clone (Fig. 1A) or with the 2.13-kb *hph-B* cassette (Fig. 1B). Western blot analysis of the proteins secreted from Cm-PL-3 and Cm-PL-10 into pectolytic enzyme-inducing media (PEIM; Prusky et al. 1989) revealed the presence of two bands of 39 and 41.5 kDa, corresponding to the PL bands in Cg-14 and L-2.5, respectively (Fig. 2).

PL activity of the proteins secreted into PEIM was measured as described by Collmer et al. (1988). Four days after

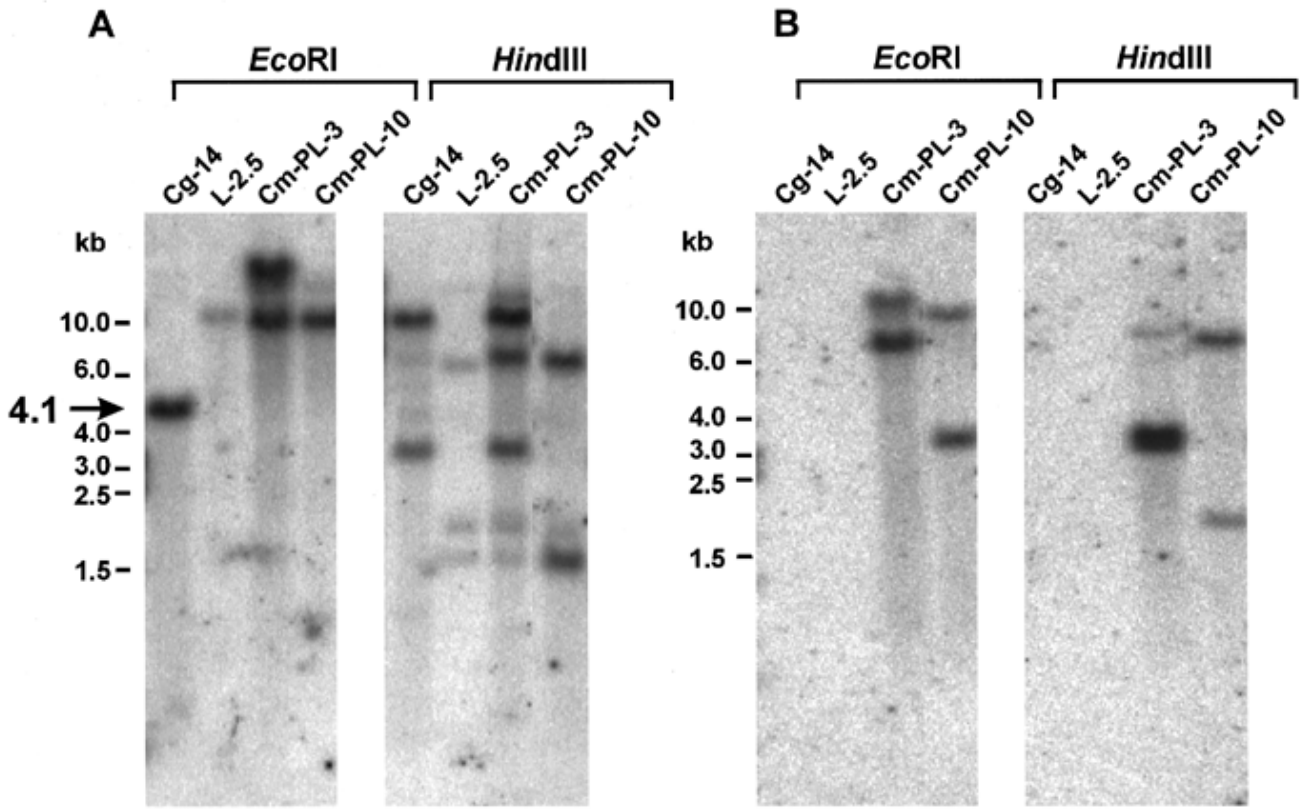

Fig. 1. Southern blot analysis of the transformed isolates, Cm-PL-3 and Cm-PL-10, compared with wild-type isolate Cg-14 of *Colletotrichum gloeosporioides* and wild-type isolate L-2.5 of *C. magna*. Fungal DNA was extracted as described by Rodriguez (1993) from 1 g of dry mycelium. Fungal DNA (5 µg) was restricted for 15 h with 10 U of *Eco*RI or *Hin*dIII restriction enzymes (Promega, Madison, WI). Restricted DNA was subjected to Southern blot (Sambrook et al. 1989) onto a HyBond$^+$ nylon membrane (Amersham, Buckinghamshire, UK). All hybridizations were carried out at 65°C and washes were with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Membrane was probed with (**A**) 4.1-kb *pel* or (**B**) 2.13-kb hph-B.

isolate growth, PL activity was 3.1×10^{-3} U/min for *C. gloeosporioides*, and 4.1×10^{-3} U/min for *C. magna*. Increased activity, reaching 168 and 231% of the wild-type *C. magna* isolate L-2.5, was observed in Cm-PL-3 and Cm-PL-10, respectively. Activity was calculated from each isolate's regression line formula ($r^2 = 0.999$, for all four isolates). All PL activities were significantly different ($P < 0.01$) between the different isolates. PL activity was additive for the transformed isolate Cm-PL-3 and significantly more than additive for Cm-PL-10, relative to the two wild-type isolates Cg-14 and L-2.5. Although both isolates were transformed with the same gene, PL activity was significantly greater for transformed isolate Cm-PL-10 than for Cm-PL-3. The differences in activities may be explained by the different patterns of gene integration into the fungal genome, observed by Southern blot analysis, which may have affected the expression of the integrated *pel*, as has been described for higher plants and fungi (MacKenzie et al. 1993).

Germinated conidia of wild-type *C. magna* isolate L-2.5 produced appressoria that breached the cuticle of the avocado fruit (cv. Fuerte) but caused only a 2-mm-diameter lesion after 5 days (Fig. 3A). Decay caused by Cg-14 was fourfold larger in diameter than that caused by L-2.5 (Fig. 3A). Transformed isolates Cm-PL-3 and Cm-PL-10 showed a 1.8-fold increase in decay, compared with L-2.5 (Fig. 3B). Inoculation of avocado mesocarp with the different isolates showed no difference in maceration development (data not presented). The fact that the transformed isolates were more aggressive on avocado pericarp, but that these differences were eliminated when inoculation was carried out in the avocado mesocarp, further

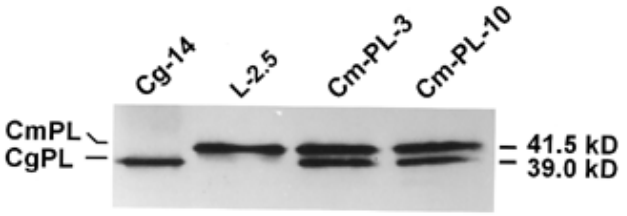

Fig. 2. Pectate lyase (PL) secretion from the wild-type isolates Cg-14 and L-2.5, and the transformed isolates, Cm-PL-3 and Cm-PL-10. Isolates were grown for 4 days in pectolytic enzyme-inducing media (PEIM). Clear supernatant (100 ml) was concentrated by rotavapor (Buchii, Flawil, Switzerland) at 42°C and samples (10 µl) were subjected to Western blot (immunoblot) analysis by running on a 12.5% SDS (sodium dodecyl sulfate)-polyacrylamide gel (Mini-Protean II, Bio-Rad Laboratories, Hercules, CA) for 1 h at 150 V constant voltage and transferring to a nitrocellulose membrane (Pall Gelman Sciences, Northampton, UK), which was analyzed with PL antibodies as described by Wattad et al. (1995).

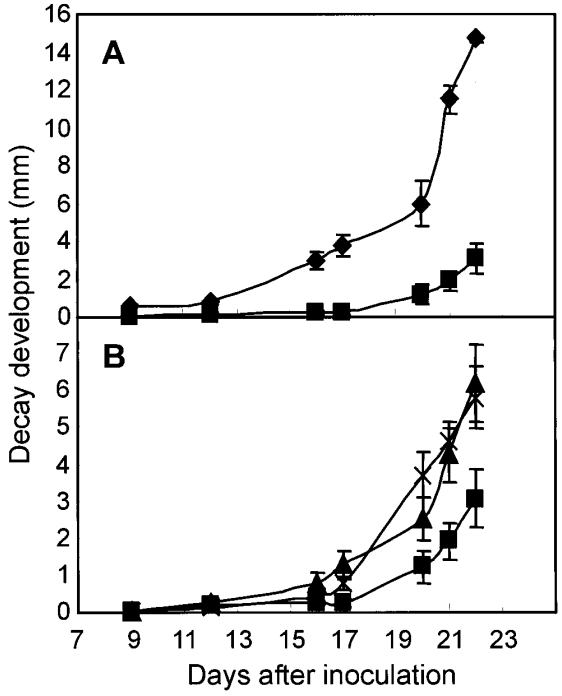

Fig. 3. Decay development on avocado fruit cv. Fuerte. Freshly harvested avocado fruits (20 fruits) were inoculated with (**A**) a 10-µl conidia suspension (1×10^6 conidia per ml) of Cg-14 (◆) or L-2.5 (■), or with (**B**) L-2.5 (■), Cm-PL-3 (▲) or Cm-PL-10 (**X**). Isolates were spot-inoculated twice and compared with Cg-14 inoculation on the opposite side of the same fruit. Decay diameter was monitored during fruit storage at 20°C. Results show one representative experiment out of three independent experiments and the standard error.

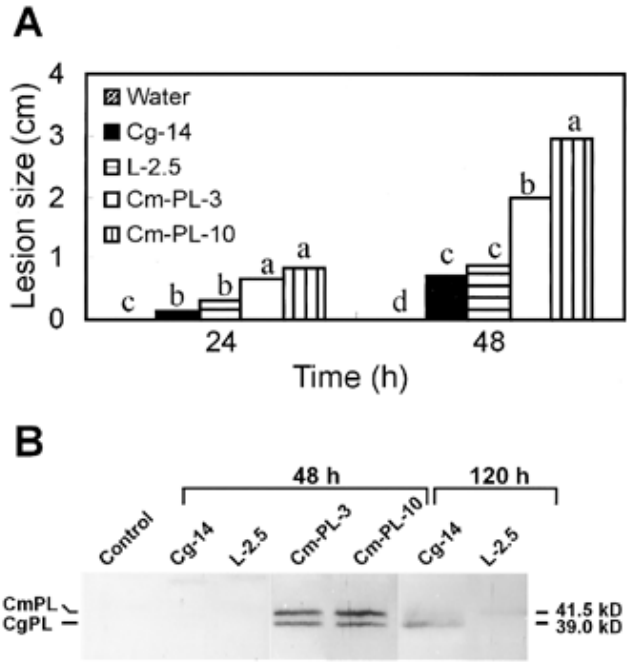

Fig. 4. Lesion development on detached watermelon seedling stems (cv. Odem) formed by Cg-14, L-2.5, Cm-PL-3, Cm-PL-10, or water. Detached, 10-day-old watermelon (cv. Odem) seedling stems were wounded by pin-pricking and inoculated with 5 µl per stem of each of the different isolates at 4×10^6 conidia per ml. **A,** Extent of tissue colonization was measured 24 and 48 h post inoculation. **B,** Pectate lyase (PL) from inoculated stem tissue 48 and 120 h post inoculation was detected on eight inoculated stems per isolate, and a water control. Stems were maintained under fluorescent light at 20°C in 90% humidity for 120 h. Samples were homogenized when the whole 5-cm stem sections were macerated and 10 µg of protein was loaded for Western blot (immunoblot) analysis. All experiments were conducted three times. **A,** Letters above bars represent significant differences between isolates ($P < 0.01$) according to analysis of variance.

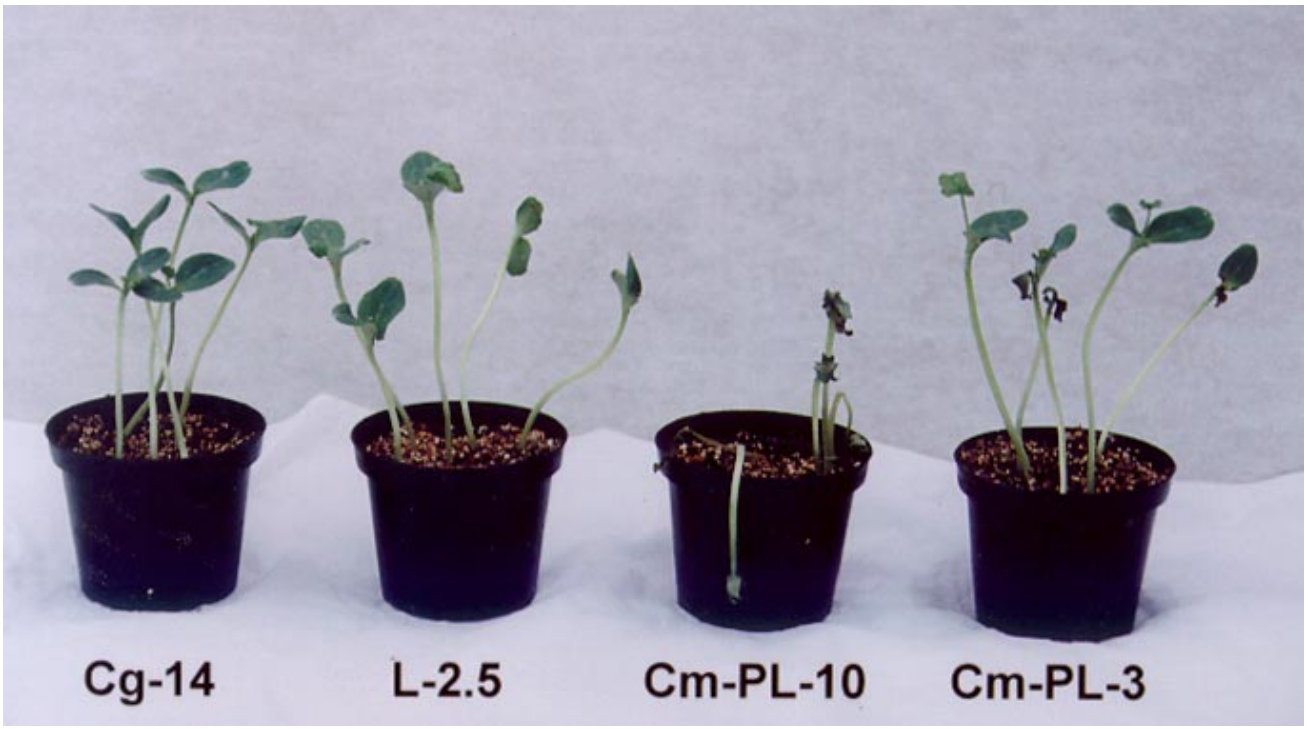

Fig. 5. Symptoms of necrotic development and damping off on watermelon seedlings (cv. Odem) caused by Cg-14, L-2.5, Cm-PL-3, or Cm-PL-10. Ten-day-old watermelon seedlings were inoculated on the apex with 20 µl of conidial suspension at a concentration of 4×10^6 conidia per ml of each different isolate. Plants were maintained in a greenhouse at 25°C in 90% humidity for 48 h. Water inoculation was used as a control. Pathogenicity was observed 7 days after inoculation as developing necrosis and damping off.

supports the importance of PL for the initial stages of fungal host colonization.

When detached watermelon seedling stems were inoculated, both transformed isolates caused significantly larger lesion development 24 and 48 h post inoculation (Fig. 4A). PL secretion during the colonization of watermelon seedling stems by Cm-PL-3 and Cm-PL-10 was detected 48 h post inoculation, for Cg-14 and L-2.5 only after 120 h (Fig. 4B). Inoculation of watermelon seedlings with Cm-PL-10 and Cm-PL-3 caused damping off and leaf necrosis, whereas no symptoms developed with the two wild-type isolates Cg-14 and L-2.5, 6 days post inoculation (Fig. 5). The initial symptoms on the seedlings were soft maceration, indicating the activity of pectin-degrading enzymes. The increase in PL activity accompanied by the detection of both PLs in the tissue 48 h after inoculation, in contrast to 120 h in the two wild-type isolates, suggests the involvement of PL in tissue maceration. Since Cg-14 secretes PL but causes much-reduced symptoms on detached watermelon, we assume that the combination of the transformed isolate with the new introduced PL increased its aggressiveness on the watermelon host. Various explanations may be considered as a basis for increased aggressiveness: the expressed PL promotes improved penetration of the stem vessels (Freeman and Rodriguez 1993) and during colonization enables more efficient expression of other virulence factors. It may also be that PL levels above the basic threshold of L-2.5 enable enhanced colonization of the plant tissue. It is clear from symptom development that the introduced PL affects the basic compatibility response of the watermelon seedlings.

Until now, most reports on the disruption of a single CWDE or combinations of CWDEs have shown limited or no contribution to the pathogenic abilities of the pathogen; for example, disruption of PNLA from *C. gloeosporioides* and PL from *Nectria haematococca* did not reduce their virulence (Bowen et al. 1995; Gou et al. 1995). Mutations in all known *pel* genes of *Erwinia chrysanthemi* reduced its virulence but the bacterium was still able to macerate the tissue (Ried and Collmer 1988), suggesting that a second set of PLs were activated (Kelemu and Collmer 1993). Gene disruption of the two endo-β-1,4-xylanases from *M. grisea* had no effect on pathogenicity and revealed the secretion of an additional xylanase enzyme (Sheng-Cheng et al. 1997).

Our system further elucidates, by heterologous expression, the importance of PL as a pathogenicity and virulence factor (penetration and colonization) in watermelon seedlings and as a virulence factor (colonization) in avocado fruits. The addition of PL with its native control system demonstrated differences in attack between hosts and on the same host depending on the assay. This supports heterologous expression as a tool for determining the importance of specific genes in pathogenicity, and at the same time demonstrates the complexity of a system that requires more than one factor to express the pronounced contribution of a single enzyme to fungal pathogenicity.

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